The second international standard for tetracycline *

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A second international standard has been established to replace the first International Standard for Tetracycline, stocks of which were depleted. The International Unit of tetracycline is defined as the activity contained in 0.00101833 mg of the second International Standard, corresponding to a potency of 982 IU/mg. These values were assigned on the basis of the results of an international collaborative assay carried out by laboratories in 19 countries. Most of the assays were by diffusion techniques ; a few were by turbidimetric methods. Both procedures gave statistically valid assays but there was some heterogeneity of repeated estimations within a laboratory and also some heterogeneity of the mean estimates from each laboratory. The agreed defined potency was the unweighted mean of 281 individual assays; the confidence limits were 978–986 IU/mg.

Since in 1969 stocks of the first International Standard for Tetracycline were becoming depleted, material suitable to serve as a second international standard was obtained and a collaborative assay arranged. The sample, consisting of approximately 430 g of tetracycline hydrochloride, was made available through the generosity of The Pfizer Group, United Kingdom, and through the good offices of Mr E. Addison of that company. In October 1969 the WHO Expert Committee on Biological Standardization (1970) authorized the National Institute for Medical Research, London, to establish this material as the second International Standard for Tetracycline on the basis of the results of the collaborative assay and to define the international unit with the agreement of the participants.

THE PROPOSED SECOND INTERNATIONAL STANDARD FOR TETRACYCLINE

The sample of tetracycline hydrochloride was received at the National Institute for Medical Research, London, in a single container in May 1969. The following analytical data were supplied by the manufacturer:

lot No. 903–7172	
microbiological assay [3]	977 " μg "/mg
chemical assay	974 μg/mg
volatile matter	0.48%
sulfated ash	0.01%
chromatography:	
anhydrotetracycline	0.7%
4-epitetracycline	1.5%

The sample was kept in its original sealed container in the dark at −20°C until later in May 1969, when the material was mixed and distributed into approximately 5 000 nonactinic, neutral glass ampoules so that each contained approximately 75 mg. The ampoules were fitted with vented polyethylene plugs and dried over phosphorus(V) oxide *in vacuo* to constant weight in 7 days. After filling with pure dry nitrogen, the ampoules were sealed by fusion of the glass, tested for leaks, and stored in the dark at −20°C.

The material in the ampoules was examined by heating at 60°C over phosphorus(V) oxide at a pressure of <0.05 mmHg for 11 hours; no significant loss of weight was observed.

When exposed to the atmosphere at 60% relative humidity in an open weighing bottle, a sample of the material in the ampoules increased in weight by approximately 0.1% in 3 hours.

[3] When μg is placed between quotation marks (" μg "), it refers to a certain amount of activity (potency) and not to its accepted usage as a unit of mass.

Table 1. Details and results of bioassays used by participating laboratories

Laboratory No.	Assay method	Design	Test organism	No. of assays	Mean potency (IU/mg)	Heterogeneity	Total weight	Average weight/ assay
1	Petri dish	3 + 3	*Bacillus subtilis* ATCC 6633	9	993	N.S.	59 969	6 663
2	Petri dish	3 + 3	*Bacillus subtilis* ATCC 6633	8	993	N.S.	277 256	34 657
2	large plate	2 + 2	*Bacillus pumilus* ATCC 8241	10	986	N.S.	1 487 861	148 786
3	Petri dish (6 assays) large plate (3 assays)	3 + 3	*Sarcina lutea* ATCC 9341	9	1 004	N.S.	55 477	6 164
3	Petri dish (4 assays) large plate (3 assays)	3 + 3	*Bacillus pumilus* NCTC 8241	7	984	N.S.	18 456	2 637
4	Petri dish	3 + 3	*Bacillus subtilis* var. L2 No. 21	18	975	N.S.	182 650	10 147
5	turbidimetric	3 + 3	*Staphylococcus aureus* ATCC 6538-P	6	969	$P<0.001$	13 720	2 287
6	Petri dish	2 + 2	*Sarcina lutea* ATCC 9341	24	986	N.S.	42 703	1 779
7	Petri dish	3 + 3	*Sarcina lutea* ATCC 9341	12	977	N.S.	155 400	12 950
8	large plate	3 + 3	*Sarcina lutea* NCTC 8340	4	993	$P<0.01$	11 878	2 970
8	large plate	3 + 3	*Staphylococcus aureus* TH6	3	983	$P<0.01$	6 671	2 224
9	large plate	3 + 3 (4 assays) 2 + 2 20 assays)	*Bacillus cereus* var. *mycoides* ATCC 11778	24	978	N.S.	543 759	22 657
11	turbidimetric	3 + 3 (1 assay) 2 + 2 (3 assays)	*Staphylococcus aureus* ATCC 6538-P	4	953	N.S.	30 773	7 693
12	large plate	3 + 3	*Bacillus pumilus* NCTC 8241	11	1 001	N.S.	321 109	29 192
13	turbidimetric	6 + 6	*Staphylococcus aureus* ATCC 6538-P	11	1 044	$P<0.001$	25 317	2 302
14	large plate	3 + 3 (6 assays) 2 + 2 (22 assays)	*Bacillus subtilis* No. 201	28	991	$P<0.001$	332 979	11 892
15	large plate	3 + 3	*Bacillus subtilis* ATCC 6633	14	950	N.S	89 315	6 380
15	large plate	3 + 3	*Bacillus cereus* ATCC 9634	1	1 054	—	4 754	4 754
17	Petri dish	3 + 3	*Bacillus subtilis*	15	969	$P<0.01$	77 900	5 193
17	Petri dish	3 + 3	*Sarcina lutea* ATCC 9341	8	970	N.S.	109 073	13 634
18	turbidimetric	7 + 2	*Staphylococcus aureus* ATCC 6538-P	5	923	$P<0.02$	5 757	1 151
18	Petri dish	2 + 2	*Bacillus cereus* var. *mycoides* ATCC 11778	4	959	N.S.	8 018	2 005
18	Petri dish	2 + 2	*Sarcina lutea* ATCC 9341	5	991	N.S.	4 494	899
20	Petri dish	4 + 4	*Bacillus cereus* ATCC 9634	10	982	$P<0.001$	32 088	3 209
21	large plate	4 + 4	*Sarcina lutea* ATCC 9341	1	946	—	6 416	6 416
21	large plate	4 + 4	*Bacillus cereus* var. *mycoides* ATCC 11778	9	974	N.S.	154 471	17 163
21	large plate	4 + 4	*Bacillus pumilus* NCTC 8241	4	980	N.S.	16 521	4 130
21	turbidimetric	4 + 4	*Klebsiella pneumoniae* ATCC 10031	5	962	$P<0.001$	20 899	4 180
22	large plate	3 + 3	*Bacillus cereus* NCIB 8122	4	987	N.S.	174 608	43 652
23	Petri dish	3 + 3	*Bacillus pumilus* NCTC 8241	4	1 006	N.S.	200 511	50 128
23	Petri dish	3 + 3	*Bacillus cereus* NCTC 10320	4	995	N.S.	94 254	23 564

THE FIRST INTERNATIONAL STANDARD
FOR TETRACYCLINE

This was established in 1957 (WHO Expert Committee on Biological Standardization, 1958) and has been described by Humphrey et al. (1957). Its potency is 990 IU/mg.

THE COLLABORATIVE ASSAY

The participating laboratories were asked to estimate the potency of the proposed second international standard for tetracycline in terms of the International Standard for Tetracycline by biological assay. They were supplied with 5 ampoules of each of the two materials. As is customary in these collaborative assays, laboratories were asked to design their assays in such a way that each assay would contain sufficient information to provide from its own internal evidence an estimate of potency and the fiducial limits to that estimate. It was also requested that the assays should provide data to allow evaluation of linearity and parallelism of the log dose–response curves. The dilutions of the standard and unknown, which formed the basis of the assay, were to be tested with or without replication in a single unit of the experiment.

Participants were asked to provide additional information by comparing the two materials by any chemical method with which they were familiar.

Altogether 23 laboratories in 21 countries agreed to take part in the collaborative assay, and assay results were received from 21 laboratories, one of which submitted results from chemical assays only. In this report the laboratories are referred to by number only, the number not necessarily bearing any relation to the order in which the laboratories are listed in Annex 1.

STATISTICAL ANALYSIS

In all, 281 assays were carried out by biological methods and 33 by chemical methods. The biological assays were carried out using either the plate diffusion method (250 assays) or the turbidimetric method (31 assays) and with one of six different test organisms. Table 1 lists, for each laboratory, the number of assays and the method and test organism used. The chemical assays are reported in Table 2.

In accordance with the usual practice, only the biological assays have been used to determine the potency of the proposed second international standard.

For each assay, the log potency was estimated

Table 2. Details and results of chemical assays reported by participating laboratories

Laboratory No.	Method	No. of assays	Mean potency (μg/mg)
1	colorimetric [a]	4	976
	spectrophotometric [b]	4	981
10	colorimetric [a]	4	983
	spectrophotometric	4	975
11	spectrophotometric [b]	2	993
15	spectrophotometric [b]	1	983
17	spectrophotometric [b]	4	999
20	colorimetric [c]	10	988
All assays		33	985

[a] Iron(III) chloride method.
[b] Ultraviolet absorption.
[c] Acid colorimetric method: for further details of the methods see, for example, Grove & Randall (1955).

from the horizontal distance between the two log dose–response lines, an analysis of variance appropriate to the design of the assay was calculated, and statistical tests of significance for parallelism and curvature were carried out. For Laboratories 1, 4, and 9, the square of the zone diameter was used as the response metameter, since this procedure improved linearity and parallelism. In analysing the assay data for the turbidimetric assays from Laboratories 13 and 18, data relating to some dose levels were omitted from the statistical analysis; both laboratories had used a large number of dose levels (Laboratory 13 used a 6+6 design, Laboratory 18 a 7+2 design). It was found that the log dose–response lines were not linear over their entire length. A graph of response against log dose was plotted for each assay and the data that were considered to relate to the straight portion of the line were included in the statistical analysis. Laboratory 21 used a 4+4 assay design, but for four diffusion assays did not submit data for all dose levels, omitting those data that it was felt would lead to curvature. In the assays from Laboratory 1, which were 3+3 block assays, the responses had been measured independently by each of three operators, but for most assays it was not possible to correlate, for a given zone diameter, the three replicate readings with the three operators since the necessary information had not been recorded. The nine assays

from this laboratory were based on three sets of weighings of the test material and of the standard, each set being assayed by each of the three operators, and the mean potency that is reported (Table 1) was obtained by analysing the readings made by the operators who actually performed the assay.

RESULTS

Statistically significant departures from parallelism of the log dose–response lines were found in four assays, a number no greater than would be expected by chance, and these particular assays were not rejected. No laboratory found the two preparations to produce log dose–response lines that were consistently concave or consistently convex, nor was the slope of the line for one preparation consistently greater than that for the other preparation.

For each laboratory, the residual mean squares from the analysis of variance were pooled over assays of the same design that used the same test organism. These pooled variances were then used within that laboratory, when weights were calculated for the log potencies from the assays from which the pooled variances had been derived. Using these weights, tests of homogeneity were made within laboratories on the log potencies of assay that had employed the same method and test organism. If the log potencies were homogeneous, the weighted mean was calculated and its weight was taken as the sum of the individual weights; if the log potencies were heterogeneous an unweighted mean was calculated and its weight assigned from the variability between the unweighted log potencies. The results from these calculations are set out in Table 1, where it can be seen that 20 laboratories provided 31 estimates of the potency of the proposed second international standard.

These 31 potencies were found to be significantly heterogeneous ($\chi^2 = 128.4$, d.f. $= 30$, P < 0.001) and the possible sources of this heterogeneity were examined. The 26 potencies obtained using the plate (diffusion) method were a heterogeneous group, as were the 5 mean potencies for the turbidimetric method (P < 0.001 for both methods), and the unweighted means were very similar—982 IU/mg for 250 plate assays, 984 IU/mg for 31 turbidimetric assays. The results in Table 1 were also tested to see whether use of a common test organism had led to greater consistency in the results from a group of laboratories. For the plate diffusion assays, the

results derived from the four test organisms that had been used by more than one laboratory (*Bacillus subtilis*, *Bacillus pumilus*, *Bacillus cereus*, and *Sarcina lutea*) were tested for heterogeneity, within organisms, between laboratories; but this was found to be no less than that between organisms. Four of the five laboratories that performed turbidimetric assays employed the same test organism (*Staphylococcus aureus*) so that it was not possible to examine such effects in this case.

In combining the results to determine the potency of the second international standard the unweighted mean of all 281 assays was taken and confidence limits were based on the variability between these assays. The mean log potency obtained in this way was 2.992, equivalent to a potency of 982 IU/mg, with 95% confidence limits of 978 to 986 IU/mg.

Although the overall mean potencies obtained by the two methods (plate diffusion and turbidimetric) were in close agreement, the data from the study indicate that turbidimetric methods gave results that were more variable, both among laboratories and among assays within a laboratory, than those obtained using plate diffusion methods. The five mean potencies obtained with turbidimetric methods range from 923 to 1 044 IU/mg, in contrast to the narrower range of 950 to 1 006 IU/mg for the 24 mean potencies obtained using plate diffusion methods (Table 1). Heterogeneity within laboratories was also more common for turbidimetric assays (4 out of 5) than for diffusion assays (5 out of 26). This is reflected in the average weight per assay; from Table 1 it can be seen that, in general, the plate assays have a higher average weight than the turbidimetric assays.

Assays from Laboratory 1

The data from Laboratory 1 were examined, for the one set where this was possible, to see whether there was any effect related to the fact that different operators assayed the same solutions, or read the same plate. The following observations were made.

(1) When the three operators (A, B, and C) read the same plate, A and C obtained virtually identical readings (zone diameters), but B's readings were consistently higher, the average difference being as high as 7% in some assays.

(2) Probably because these differences in reading the zone diameters were consistent, there were no significant differences in the potencies calculated from the readings of the different operators. When each operator's readings were analysed as separate

assays, the three potencies varied by as much as 4.5% in some cases but the estimates were not consistently different over all assays. Mean values of 996 IU/mg for A, 988 IU/mg for B, and 990 IU/mg for C were obtained.

Chemical assays

Three different chemical assay procedures were used in 6 laboratories but the mean value for all assays (Table 2) agreed closely with the mean potency obtained by biological assay.

DISCUSSION

Both intra- and inter-laboratory heterogeneity of the potency estimates was observed in this collaborative study, but the intra-laboratory variations were no greater than is usually encountered with this type of high precision assay of antibiotics. The mean estimate of potency of the second international standard was based on a greater number of assays than was used to establish the first international standard (Humphrey et al., 1957). Although there was inter-laboratory heterogeneity in both international collaborative studies, the variation about the mean of ±7.5% in the present study was much less than the corresponding range observed in the collaborative assay of the first international standard for tetracycline, i.e., ±15%.

The present results show that the internal evidence of the turbidimetric assays provided a less reliable indication of the true assay variability than did the internal evidence of the plate diffusion assays, i.e., the weight of the log potency overestimated the reproducibility between assays, particularly for turbidimetric assays. This overestimation reflects the very small variability between the measurements of replicate responses at the individual dose levels within an assay, and it seems possible that the designs,

techniques, and procedures that are commonly used in turbidimetric assays are such that the true variability of the replicate responses may not be revealed.

The chemical estimates of the purity of the two preparations were very similar: the mean for the first international standard was 987 µg/mg and that for the second international standard was 985 µg/mg. Thus the estimates of the mean potency of the second international standard determined biologically and chemically were in close agreement.

The second international standard was found to be approximately 1% less potent than the first international standard, a slight, but nevertheless significant difference. The two materials have been examined for related impurities such as chlortetracycline and tetracycline degradation products by the chromatographic methods of Addison & Clark (1963), the *British Pharmacopoeia 1968*, the *Code of Federal Regulations, 1969*, and Fernandez et al. (1969). The only detectable differences were the slightly higher content of epitetracycline in the first international standard and the presence of a small proportion of anhydrotetracycline (<1.0%) in the second international standard. Traces of epianhydrotetracycline, but no chlortetracycline, were detected in both standards.

ESTABLISHMENT OF THE SECOND INTERNATIONAL STANDARD AND DEFINITION OF THE INTERNATIONAL UNIT

In accordance with the instructions of the WHO Expert Committee on Biological Standardization (1970), the above material was established as the second International Standard for Tetracycline and with the agreement of the participants in the collaborative assay the potency was defined as 982 IU/mg. The international unit for tetracycline is defined as the activity contained in 0.00101833 mg of the second International Standard for Tetracycline.

RÉSUMÉ

DEUXIÈME ÉTALON INTERNATIONAL DE TÉTRACYCLINE

Un deuxième étalon international de tétracycline a été constitué afin de remplacer le premier étalon international dont les stocks étaient presque épuisés.

Le National Institute for Medical Research, de Londres, s'est chargé de répartir le matériel proposé en ampoules et d'organiser un titrage comparatif auquel ont participé 21 laboratoires de 19 pays. On a procédé au total à

281 essais biologiques sur six micro-organismes différents, par diffusion ou par turbidimétrie, et à 33 analyses chimiques. Seules les données fournies par les épreuves biologiques ont servi à définir l'activité.

L'analyse des résultats a fait ressortir une certaine hétérogénéité des estimations de l'activité à la fois dans un même laboratoire et entre les laboratoires. Cette

hétérogénéité n'a pu être attribuée à l'utilisation de méthodes ou de micro-organismes différents. La combinaison des résultats des 281 essais a conduit à adopter une valeur moyenne d'activité de 982 UI/mg, avec des limites de confiance de 978 à 986 UI/mg.

Conformément aux instructions du Comité OMS d'experts de la Standardisation biologique (1970), les participants au titrage comparatif ont constitué le matériel proposé en deuxième étalon international de tétracycline dont l'activité a été fixée à 982 UI/mg. L'unité internationale de tétracycline a été définie comme l'activité de 0,00101833 mg du deuxième étalon international de tétracycline.

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Annex 1

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